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Degradation of orange dyes and carbamazepine by Soybean Peroxidase immobilized on silica monoliths and titanium dioxide

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Abstract

In this paper, the removal of three common dyes (Orange I, Orange II and Methylorange) and of the anticonvulsant drug carbamazepine from aqueous solutions by means of enzymatic and photocatalytic treatment was studied. Soybean Peroxidase (SBP) was used as biocatalyst, both free in solution and immobilized on silica monoliths, and titanium dioxide as photocatalyst. The combination of the two catalysts led to a faster (about 2-4 times) removal of all the orange dyes compared to the single systems. All the dyes were completely removed within 2 hours, also in the presence of immobilized SBP. As for carbamazepine, photocatalytic treatment prevail on the enzymatic degradation, but the synergistic effect of two catalysts led to a more efficient degradation; carbamazepine complete disappearance was achieved within 60 min with combined system, while up to 2 h are required with titanium dioxide only.

Keywords

Soybean peroxidase; azo dye; orange; methylorange; immobilization; titanium dioxide; carbamazepine.

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Introduction

Removal of organic pollutants from urban and industrial wastewaters is one of the main topic in modern research and different methods have been proposed to efficiently and inexpensively solve the problem. Indeed, several chemical-physical methods, such as coagulation, flocculation, adsorption, electrochemical and Advanced Oxidation Processes (AOP), were studied and commonly applied to remove water pollutants (Martínez-Huitle and Brillas 2009; Gupta and Suhas 2009; Khin et al. 2012; Kalsoom et al. 2012; Oturan and Aaron 2014; Mehrjouei et al. 2015). Moreover, in modern plants these methods are usually coupled with biological treatments by means of active sludge which allow to obtain the almost complete transformation of the organic matter in CO_2 (Saratale et al. 2011; Sarayu and Sandhya 2012; Ozgun et al. 2013). Sequential chemical-biological treatments are generally effective in the degradation of organic pollutants but, as recently reviewed by Guieysse and Norvill (Guieysse and Norvill 2014), the whole process has to be evaluated and adapted to specific situations, in order to obtain the complete removal of pollutants.

In the last decades new chemicals were detected in wastewaters as a results of new industrial processes, and increased consumption of pharmaceuticals and personal care products (PPCPs) (Stuart et al. 2012; Santos et al. 2013; Richardson and Ternes 2014; Arpin-Pont et al. 2016). Some of these emerging contaminants are very stable in water and recalcitrant to

degradation with the common treatments, so exercising for long periods an adverse effect on the environment. Therefore, new strategies are needed to assess the quality of water treatments, and new methods are required to unravel the problem of recalcitrant pollutants (Prasse et al. 2015).

In addition to chemical and biological methods (Chengalroyen and Dabbs 2013), the use of isolated enzymes has been proposed for the degradation of pollutants, in particular in the case of bleaching of azo dyes wastes (Singh et al. 2015). Laccases and peroxidases, both from microorganism or plant, have been successful employed in the oxidative decolorization of several dyes and other pollutants (Husain 2010; Strong and Claus 2011; Ramirez-Montoya et al. 2015; Kalsoom et al. 2015).

Enzymatic processes allow to exploit the potential of enzymes as biological catalyst avoiding the problems arising from the need to keep alive the microorganisms from which they derive. On the other hand, in the use of isolated enzymes other problems arise, such as the need to prevent their inactivation, their selective activity and their higher cost.

In order to enhance enzymes stability and reduce the operating costs, several methods of immobilization have been proposed and applied in many different conditions (Garcia-Galan et al. 2011; DiCosimo et al. 2013; Sheldon and Van Pelt 2013). However, immobilization methods generally induce conformational changes in the enzymatic structure that can result in a lower catalytic efficiency. In the case of peroxidases, the balance between the gain in stability and reuse and the loss of activity is generally positive and allowed to use these systems in different biotechnological applications (Longoria et al. 2010; Husain 2010).

Furthermore, the combination of different treatment, such as AOP and biological treatments, has been proven effective in complete removal of various type of pollutants (Oller et al. 2011). In a previous work, the synergistic effect of TiO_2 and commercial Soybean Peroxidase (SBP) in the degradation of 2,4,6-trichlorophenol was observed (Calza et al. 2014). SBP is a monomeric

Fe(III)-heme peroxidase (Henriksen et al. 2001) that catalyzes the oxidation of inorganic and organic substrates by hydrogen peroxide and has high resistance to thermic and chemical denaturation (Kamal and Behere 2002; Boscolo et al. 2006). These properties make SBP particularly suitable for industrial applications, and particularly useful in the degradation of dyes (Marchis et al. 2011; Kalsoom et al. 2013; Silva et al. 2013).

In this work we extend the studies on the TiO_2/SBP system to the degradation of three common orange dyes and a recalcitrant pollutant, carbamazepine, by means of SBP extracted by soybean hulls in our lab and immobilized on silica monoliths. Single pieces of porous silica were previously prepared with different techniques and used as support for enzymes to obtain the solid phase of separative columns (Magner 2013). For our purposes, monoliths were obtained by stable aggregation of silica particles in the presence of bio-based soluble organic substances extracted by composted urban waste and successively functionalized with SBP by using a previously reported method (Magnacca et al. 2012).

Materials and Methods

Materials

HPLC grade water was obtained from MilliQ System Academic (Waters, Millipore). HPLC grade acetonitrile (BDH) was filtered through a 0.45 μm filter before use. Experiments were carried out using TiO_2 P25 Evonik as the photocatalyst. Orange I (4-(4-Hydroxy-1-naphthylazo)benzenesulfonic acid sodium salt), orange II (4-(2-Hydroxy-1-naphthylazo)benzenesulfonic acid sodium salt), methylorange (4-(4-Dimethylaminophenylazo)benzenesulfonic acid sodium salt) and carbamazepine were purchased by Aldrich.

SBP purification

Soybean peroxidase was extracted from hulls of fresh soybean (*Glycine max*) seeds and successively purified by means of the following procedure:

a. 100 g of seed hulls were added to 400mL of phosphate buffer 0.025 M at pH 7, left under stirring for 30 min at room temperature and then separated from the solution by filtration with gauze. The treatment was repeated until the resulting solution gave a negative response to peroxidase activity test with the H_2O_2/DMAB-MBTH system (Ngo and Lenhoff 1980).
b. The SBP containing solutions were combined and concentrated by a Vivaflow 50 (Sartorius, 30,000 MWCO) tangential filter. The proteins were then precipitated by addition of ammonium sulphate until saturation (53 g/100 mL) and the mixture was left under stirring for one night at 4 °C.
c. The precipitate was centrifuged for 20 min at 4,000 rpm and dissolved in 250 mL of phosphate buffer 0.025 M pH 7. The resulting solution was then dialyzed for 24 h at 4 °C against the same buffer.
d. The dialyzed fraction was loaded onto a column (4 cm × 20 cm) containing DEAE-Sepharose CL-6B (Sigma-Aldrich) ionic exchange resin, washed with three volumes of phosphate buffer 0.025 M pH 7, and eluted with a KCl gradient 0 – 0.5 M in the same buffer. The fractions were collected, analyzed by means of UV-visible spectroscopy and selected on the basis of their RZ values (Reinheitszahl, RZ = Abs_{403nm}/Abs_{280nm}).
e. The selected fractions were pooled and concentrated by ultrafiltration on Vivaspin 20 (Sartorius, 10,000 MWCO) until their concentration was approximately 0.100 mM. The final SBP sample was then stored frozen at –12 °C until use.

Monoliths synthesis and functionalization

Silica monoliths were synthesized and functionalized with SBP by means of a previously reported procedure (Magnacca et al. 2012).

A mixture of FK320 silica powder (Degussa) dispersed in deionized water and bio-based soluble organic substances obtained by composted urban vegetable residues (Montoneri et al. 2013) was first dried at room temperature for 24 h and then heated at 500 °C to yield spherical mesoporous monoliths with a 3-4 mm diameter.

The monoliths surface was activated by reaction for 3 h at 80 °C with a 10% solution of 3-aminopropyltriethoxysilane in water. After filtration in a Buchner funnel and washing with water, 1 g of activated monoliths were suspended in a glutaraldehyde 2.5% (v/v) solution in phosphate buffer 0.1 M pH 7 and left under stirring for 1 h in the dark at room temperature. After the reaction, monoliths were washed with deionized water, added to 20 mL of SBP 0.010 mM in phosphate buffer at pH 7.5 and left to react at 4 °C for 20 h. The final product, obtained by filtration, was washed three times with deionized water and stored at 4 °C. The amount of immobilized enzyme was obtained spectrophotometrically as the difference between the initial amount of enzyme and that recovered in the washing liquids.

Irradiation procedures

The irradiations were performed by using a TL K05 UV/A lamp 25 mW/m^2 centered at 365 nm and the experiments were carried out in stirred Pyrex glass cells filled with 5 mL of sample for experiments in the presence of TiO_2 suspension/SBP, or 30 mL when using monoliths.

Orange dyes or carbamazepine were irradiated in the presence of: (1) SBP (1×10^{-8} M) and H_2O_2 (1×10^{-4} M); (2) dispersed TiO_2 (100 mg L^{-1}); (3) dispersed TiO_2 (100 mg L^{-1}) and SBP (1×10^{-8} M). The temperature reached during the irradiation was 38±2 °C. The entire content of

each cell was filtered through a 0.45 μm filter and then analyzed by the appropriate technique.

Analytical techniques

The disappearance of analytes as a function of the irradiation time was followed using an HPLC system (Merck-Hitachi L-6200 pumps), equipped with a Rheodyne injector, a RP C18 column (Lichrochart, Merck, 12.5 cm × 0.4 cm, 5 μm packing) and a UV–Vis detector (Merck Hitachi L-4200). Elution was carried out with acetonitrile and phosphate buffer (1×10^{-2} M) at pH 2.8 (40:60% v/v) at a flow rate of 1 mL/min. The analytical detector wavelength was 220 nm.

Results and discussion

Soybean extraction and purification

Several methods are reported in the literature for the extraction and purification of Soybean peroxidase from seed hulls (Gillikin and Graham 1991; Gacche et al. 2003; Bassi et al. 2004; Ghaemmaghami et al. 2010; Silva et al. 2013; Steevensz et al. 2013). We take inspiration from the literature to establish a fast and efficient purification procedure as reported in detail in the SBP purification section.

The protein was extracted from the seed hulls by several washing cycles with phosphate buffer 0.025 M at pH 7. After each wash, the supernatant was tested for peroxidase activity by the DMAB/MBTH method (Ngo and Lenhoff 1980) and the washing cycles continued until the resulting activity was negligible. At the end of the washings, the fractions were pooled, the proteins precipitated with ammonium sulfate and then dialyzed against phosphate buffer. The final purification step involved an ion exchange chromatography by a 0-0.5 M KCl gradient. Figure 1 shows the results of the protein elution: a total of 21 mg of SBP with RZ >1.2 (see inset of Figure 1) were obtained starting from 100 g of seed hulls, in agreement with

literature data (Gillikin and Graham 1991). Although the RZ value is not as high as the commercial SBP, the purification process was limited to these steps to achieve a reasonable compromise between the enzyme purity and the total yield.

Soybean immobilization

A portion of SBP was immobilized on silica monoliths (Figure 2) obtained by aggregations of silica micro particles in the presence of bio-based soluble organic substances extracted by composted urban vegetable residues as templating agent. With this procedure we were able to obtain stable monoliths with a surface area of about 120 m^2/g (Magnacca et al. 2012), suitable for the functionalization with SBP. Furthermore, the presence of mesopores with a pore size distribution centered to 35 nM (Magnacca et al. 2012) allows to functionalize all the surface of the monoliths with the protein and to facilitate an high exchange rate of substrates and products between the solution and the inner part of the monoliths.

SBP was immobilized by means of the classic APTES/glutaraldehyde method initially proposed by Weetall (Weetall 1993) and successively applied to many systems at different scale (Hartmann and Kostrov 2013; Laurenti and dos Santos Vianna Jr. 2016). The method consists in the activation of the silica surface with aminopropyl groups and the subsequent coupling with the protein by formation of imine bonds in the presence of the bi-functional reagent glutaraldehyde. The final product is active, stable and contains about 11 mg of SBP/g of monoliths, in agreement with previous results (Magnacca et al. 2012).

SBP, both in solution and immobilized, was successively used to catalyze the degradation of four different pollutants: three common azo dyes (Orange I and II, Methylorange) and the anticonvulsant drug carbamazepine (Figure 3).

Degradation of dyes

The degradation of three orange dyes was initially performed using SBP free in solution and spectrophotometrically followed by recording dyes solution spectra at different reaction times. The typical dye absorption band rapidly decreases as arose from Figure 4. The presence of isosbestic points could indicate the transformation of Orange II in a single reaction product with the rupture of the azo bond, as already reported in the case of the Horseradish Peroxidase catalyzed reactions (Zhang et al. 2013); the same trend can be observed for the degradation of Methylorange and Orange I.

The enzymatic oxidation was then coupled with a photocatalytic treatment in the presence of titanium dioxide with a double purpose: to obtain a better removal of parent molecules and to produce hydrogen peroxide through TiO_2 irradiation, in a adequate amount to supply the enzymatic reaction (Calza et al. 2014).

Figure 5 collects the disappearance profiles followed *via* HPLC for the three Orange dyes as a function of the reaction time. Figure 5 (top) shows the degradation profiles obtained with SBP free in solution. The combined TiO_2/SBP system allows to achieve half-life times of few min for all dyes and to obtain their complete degradation within 90 min. The improvement achieved with the combined system is particularly marked for Orange II degradation.

Figure 5 (bottom) reports the degradation of dyes in aqueous solution using monoliths containing the immobilized SBP. The adsorption in the dark on monoliths (without SBP) was preliminary assessed and, for all dyes, it was negligible in the investigated time window (2 hours). When using immobilized SBP, all dyes are nearly completely abated within 150 min, with the exception of Orange II, where almost 20% persists. The employment of immobilized SBP only for the degradation of the three dyes led, as expected, to a decrease in the abatement efficiency, with a rate ratio lowered from 2 (for Orange II) to 4 times (for Orange I) as assessed by the k_{obs} values in Table 1. Lastly, we test the performance of the system using both monoliths and titanium dioxide. Again, the use of a combined SBP/TiO_2 system led to an

increase in the degradation rate; in this case. Orange II was completely removed from the solution as well. The SBP immobilization led to a very slight decrease; in combined system, the constant rate ratio between SBP free and immobilized became close to 1.3 (for Methylorange and Orange II) and 2 (for Orange I) and within 2 hours of irradiation all dyes are completely degraded.

Degradation of carbamazepine

We test the system on a more refractory molecule, namely carbamazepine, and the degradation profiles over time are shown in Figure 6, while the calculated rate constant are collected in Table 1. The SBP action is limited to a 25% degradation after two hours of reaction when free in solution (Figure 6, top), confirming the difficulties in the carbamazepine removal by enzymatic treatments (Pearce et al. 2002; Lu and Uetrecht 2008). The monoliths with immobilized SBP very slowly degrade carbamazepine, with a percentage of abatement below 10% after 120 min (see Figure 6, bottom).

However, the coupling of SBP with TiO_2 revealed very promising. Although peroxidase alone is not effective in the degradation of carbamazepine, the use of SBP/TiO_2 leads to a sharp increase and permits to achieve the complete degradation in 60 min with both SBP free and immobilized on monoliths.

Conclusions

As previously reported, the combined effect of photocatalysis in the presence of titanium dioxide and SBP enzymatic action, is particularly efficient in the removal of water pollutants such as 2,4,6-trichlorophenol (Calza et al. 2014). Furthermore, the continuous production of H_2O_2 by TiO_2 irradiation endorses the enzymatic action without the external addition of

hydrogen peroxide and avoids the problem of inhibition by substrate, typical of a discontinuous supply.

In this paper we applied the same approach to the degradation of four different recalcitrant molecules. The synergistic effect of the TiO_2/SBP system was confirmed, indeed first order kinetic constants are higher for all the substrates compared to the systems with TiO_2 or SBP alone and the complete degradation is obtained in shorter times for all substrates.

The same results were obtained by using free or immobilized SBP. In general, reactions catalyzed by immobilized SBP are slower than the corresponding degradation by SBP in solution, but the possibility of reusing these catalysts makes this system more favorable for applications to real wastewaters.

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Table 1 k_{obs} (min^{-1}) calculated for the degradation of orange dyes and carbamazepine in the presence of the different catalysts

		Methylorange	Orange I	Orange II	Carbamazepine
TiO_2		0,0229	0,0366	0,0235	0,0524
SBP	free	0,0590	0,0886	0,0218	0,0182*
	immobilized	0,0169	0,0197	0,0097	0,0002
SBP/TiO_2	free	0,0595	0,1020	0,0528	0,0965
	immobilized	0,0427	0,0475	0,0405	0,0957

(*: value obtained by using only data recorded in the first 30 min of reaction)

Figure captions:

Fig 1 SBP concentration (boxes, solid line) and RZ values ($Abs_{403\ nm}/Abs_{280\ nm}$: circles, dashed line) in the protein elution from the DEAE-Sepharose column. In the inset the UV-visible spectrum of the final product

Fig 2 Silica monoliths before (A) and after (B) functionalization with SBP

Fig 3 Chemical structure of Orange I, Orange II, Methylorange and Carbamazepine

Fig 4 UV-visible spectra modifications of orange dyes during the reaction with SBP

Fig 5 Degradation of orange dyes by free or immobilized SBP and TiO_2 (boxes, solid line: TiO_2; circles, dashed line: SBP/H_2O_2; triangles, dotted line: SBP/TiO_2)

Fig 6 Degradation of carbamazepine by free or immobilized SBP and TiO_2 (boxes, solid line: TiO_2; circles, dashed line: SBP/H_2O_2; triangles, dotted line: SBP/TiO_2)

Figure 1

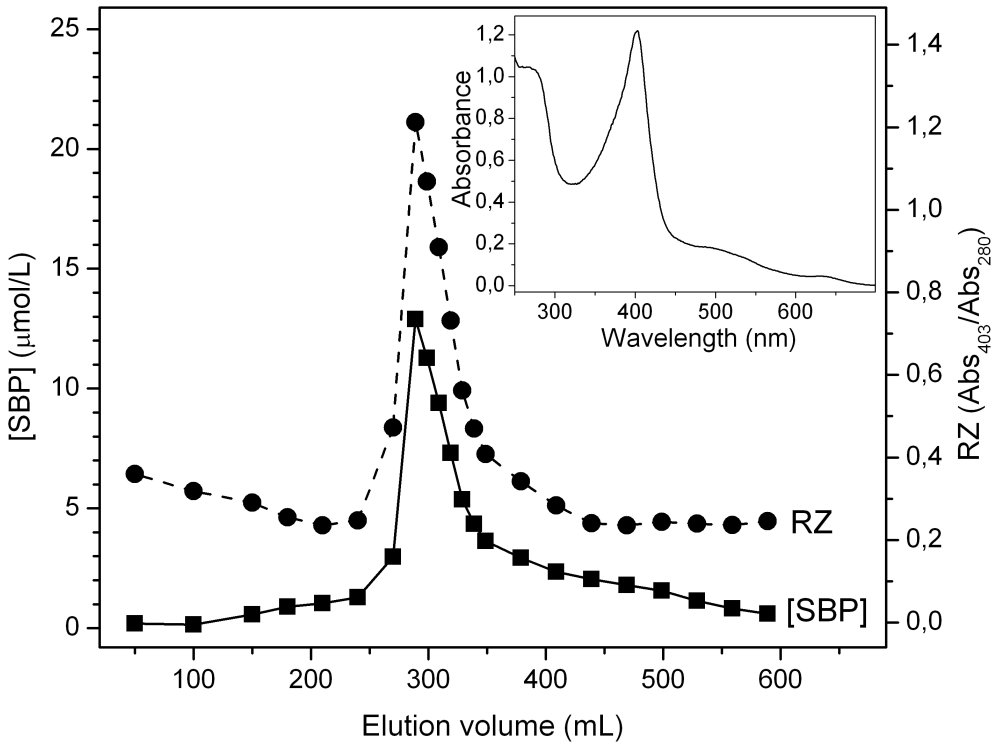

Figure 2

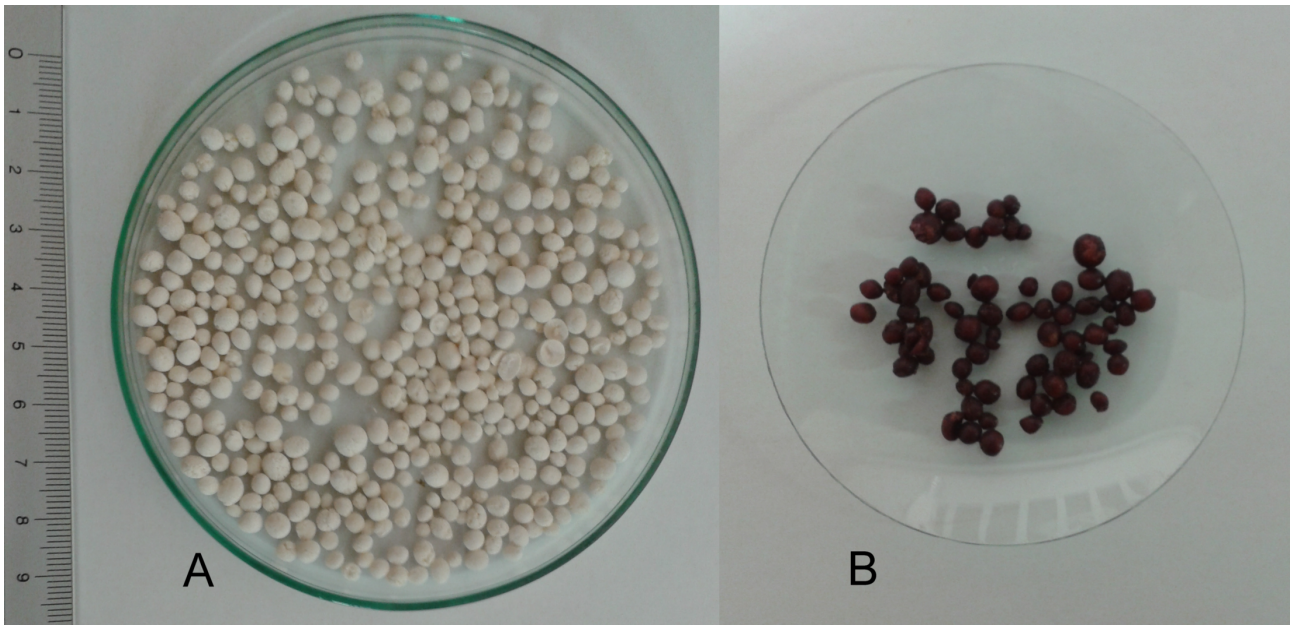

Figure 3

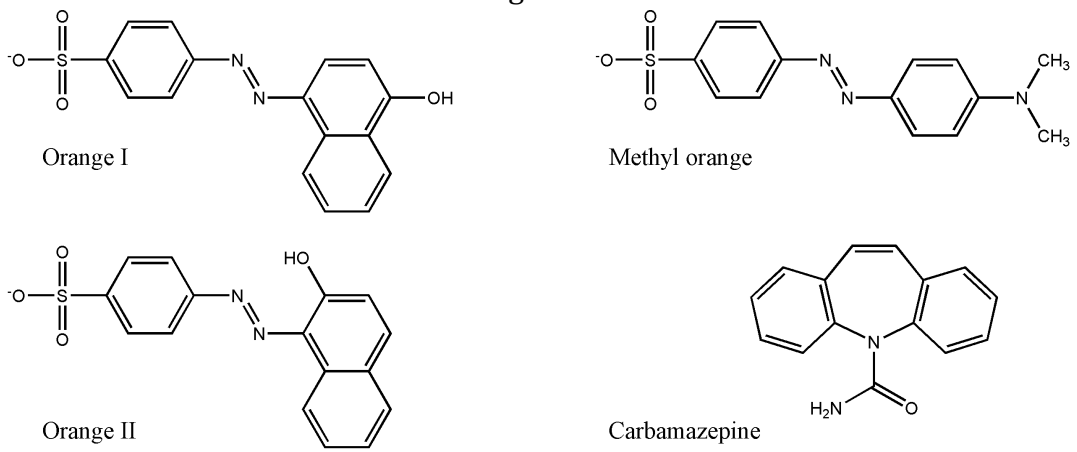

Figure 4

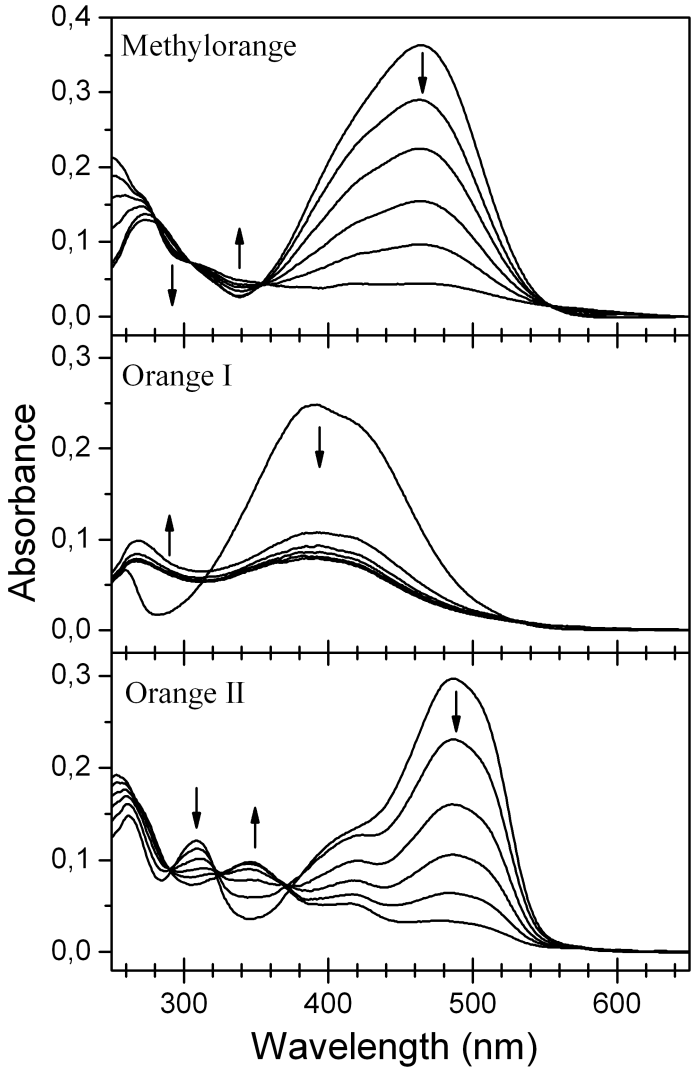

Figure 5

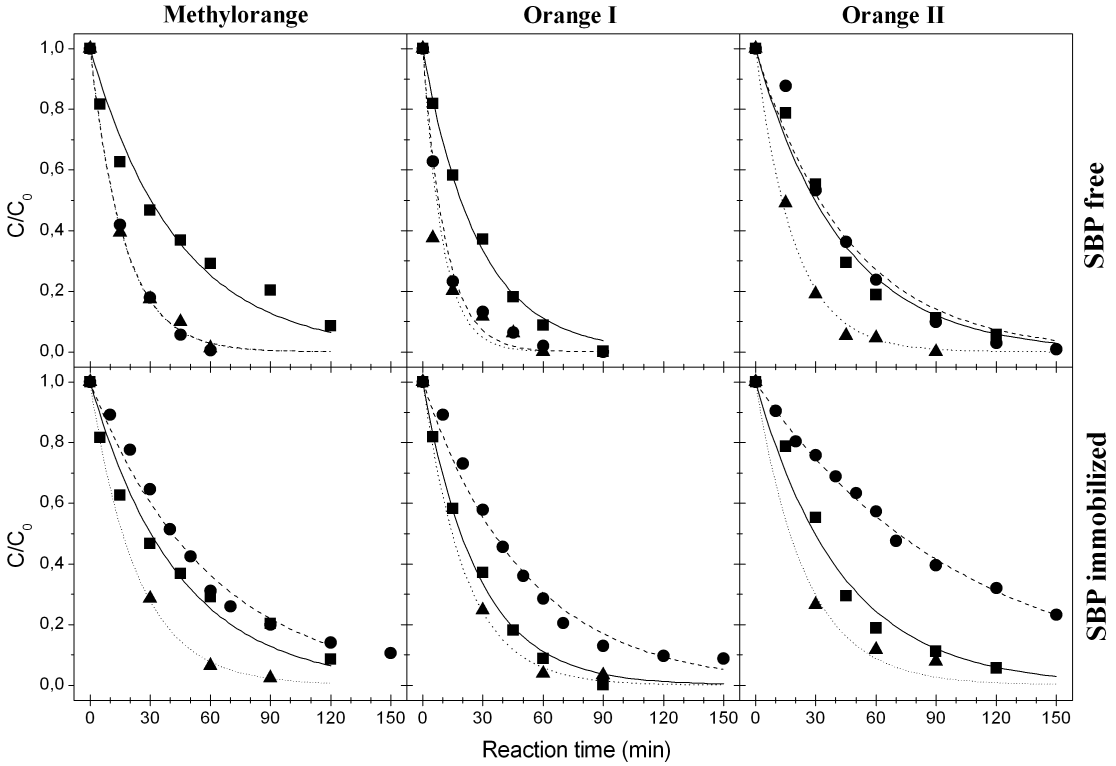

Figure 6